1993 IFSCC INTERNATIONAL CONFERENCE

Platja d'Aro, 21-23 September 1993

Costa Brava Spain

BIOCOSMETICS - SKIN AGING
1993 IFSCC BETWEEN-CONGRESS CONFERENCE
Platja d'Aro, Costa Brava, SPAIN
September 21 - 23, 1993

ANNEX 1

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BIOCOSMETICS - SKIN AGING
1993 IFSCC BETWEEN-CONGRESS CONFERENCE
Platja d'Aro, Costa Brava, SPAIN
September 21 - 23, 1993

DR.ALFONSO DE LA MAZA
INSTITUT0 DE TECNOLOGIA
QUIMICA Y TEXTIL(CSIC)
JORDI GIRONA, **18-26**
08034 BARCELONA

Barcelona, 16 Junio 1992

Apreciado Dr.De La Maza:

Acusamos recibo de su manuascrito (original y tres copias) del Artículo Titulado "STRATUM CORNEUM LÍPID LIPOSOMES:INFLUENCE OF THE LÍPID COMPOSITION ON THE BILAYER FORMATION AND THEIR PHYSICO-CHEMICAL PROPERTIES "

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BIOCOSMETICS - SKIN AGING
1993 IFSCC BETWEEN-CONGRESS CONFERENCE
Platja d'Aro, Costa Brava, SPAIN
September 21 - 23, 1993

Dr. Alfonso de la Maza
INSTITUT0 DE TECNOLOGIA
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22 de Febrero de 1993

Apreciado Dr. De la Maza:

El Comité Cientifico del 1993 IFSCC international Cosmetic Conference ha examinado cuidadosamente su abstract titulado "Stratum corneum Iipid liposomes:Influence of the lípid composition on the bilayer formation and their physico-chemical properties" y ha decidido aceptarlo para la Sesión de Posters

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Aprovechamos esta oportunidad para agrederle su participación en este importante evento.

Atentamente,

Francesc Casadó, Ph. D
Presidente
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Att.: Annex

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STRATUM CORNEUM LIPID LIPOSOMES: INFLUENCE OF THE LIPID COMPOSITION ON THE BILAYER FORMATION AND THEIR PHYSICO-CHEMICAL PROPERTIES

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ABSTRACT

The influence of different stratum comeum lipid compositions on the formation and on the physico-chemical properties of liposome vesicles has been investigated with the aim to charactenze these structures. To this end, a lipid mixture containing ceramide (40%), cholesterol (25%), palmitic acid (25%) and cholesteryl sulfate (10%) approximating the composition of stratum comeum was choosen as a central composition of the optimizing model used. From this composition, the optimizing lipid percentage range was varied for each lipid plus or minus 15% except for the cholesteryl sulfate in which case the variation was extended to plus or minus 100% due to the possible influence of this component on the physico-chemical properties of these bilayer structures. Liposomes were prepared by sonication at 80°C in a buffered medium containing 100 mM NaCl, 5 mM TRIS at pH 7.5 and supplemented in some cases with 10mM of 5-(6)Carboxyfluorescein . The mean vesicle size distnbutionand the polydispersityof liposomepreparations were determined by photon correlator spectrometry, whereas the internal volume and the bilayer permeability were determined by spectrofluorometry. The results indicated that all the lipid mixtures studied led to the formation of bilayers, their physico-chemical characteristics being dependent on the lipid composition used. increasing concentrations of ceramide, palmitic acid and cholesterol in bilayers (or low cholesteryl sulfate relative concentrations) resulted in a decreasing tendency both in the bilayer permeability as well as in the internal volume of bilayers, although with an increment both in the vesicle size distribution and the corresponding polydispersity indexes of these structures. The physico-chemical properties of the stratum comeum lipid bilayers are closely connected with a versatile range of lipid composition, the cholesteryl sulfate component playing a very important role, specially in the permeability modifications of these structures.

INTRODUCTION

The stratum corneum of mammalian epidermis contains multiple intercellular lipid membranes that are believed to constitute the epidermal barrier to water penetration. This bamer is one of the most essential properties of the stratum corneum and a number of investigations have sought the specific lipid responsible for this barrier (1,2). In this sense, Friberg (3), supported the hypothesis that the lipid barrier to water penetration of the stratum corneum is determined by the structural organization of the lipids, not by the exact chemical structure of individual species. Rowever, other authors reported the specific influence of each lipid for the maintenance of the epidernial permeability barrier (4-6).

The stratum comeum lipids have been extensively investigated for analytical composition (7-9) and for structural organization (10,11). A layered structure, according to Elias (12) is at

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present generally accepted as reflecting the organization of the lipids in the stratum comeum. This structure implies the presence of amphipathic lipids capable of appropriate orientation in the presence of water. In all cellular and intracellular membranes, such bilayers-forming lipids consist predominantly of phospholipids. However, stratum comeum has been shown to be virtually devoided of phospholipids (13,14), as a result of which its ability to form bilayers has proved somewhat surprising.

In order to establish if that stratum corneum lipids could indeed form bilayers, Wertz and Abraham (15-17) prepared liposomes from a lipid mixtures containing only ceramides (40%), cholesterol (25%), cholesteryl sulfate (10%) and free fatty acids (25%), approximating the composition of stratum comeum lipids. The results reported indicated that lipid mixtures similar to those found in stratum corneum were capable of forming unilamellar bilayers at physiologic pH. However, it could be interesting to know if there exist a range of lipid concentration able to form liposomes. Also, some physico-chemical parameters, such as permeability, vesicle size distribution etc. are needed to adequately characterize these structures. Previously we reported studies on the permeability alterations of neutral or electrically charged liposomes caused by the presence of different amphiphilic molecules at subsolubilizing concentrations (18,19). In this work we seek to determine the ability of mixtures of lipids included in the stratum comeum at different relative concentrations to form bilayers, also investigating their physico-chemical behaviour. To this end, an orthogonal composite factorial design of Box and Behnken (20) was used, the central lipid composition corresponding to the composition of stratum comeum lipids and considering a reasonable variation of plus/minus 15% for the different lipids, except cholesteryl sulfate which was varied plus/minus 100%. From these experiments some correlations between the structural organization and the composition of lipids were arisen. The results indicate that bilayer structures are formed in all cases, their physico-chemical properties being correlated with the lipid composition of vesicles. This approach can be very useful to study many posibilities of structural organization of different lipids during the differentiation process involved in the stratum corneum formation and desquamation.

EXPERIMENTAL

MATERIALS

Reagent grade organic solvents, ceramides type III (Cer), and cholesterol (Chol) were obtained from Sigma CO. (St Louis, MO). Palmitic acid (PA) (reagent grade) was obtained

from Merck (Darmstadt, Germany), and cholesteryl sulfate (Chol-sulf) was prepared by reaction of cholesterol with excess chlorosulfonic acid in pyridine and purified chromatographically. Lipids were stored in chloroform:methanol 2:1 under nitrogen at -20°C until use. Triton X-100, octylphenol polyethoxilated surfactant with 10 units of ethylene oxide and active matter of 100% was purchased from Rohm and Haas (Lyon, France).

Tris (hydroxymethyl)-aminomethane (TRIS) was supplied from Merck. The starting material 5(6)-carboxyfluorescein (CF), (Eastman Kodak, Rochester, NY) was purified according to the column chrornatographic method (21). The buffer used was 5 mM TRIS adjusted at pH 7.50 with 100mM NaCl (reagent grade) and supplemented with 10 mM CF when studying bilayer permeability of liposomes. Water was also purified by a Milli-Ro system (Millipore, Madrid, Spain).

METHODS

Preparation of large unilamellar liposomes

Liposome suspensions at different lipid compositions and constant lipid concentration (1 mg lipid/ml) were prepared following the method described by Wertz and Abraham (15).

Individuai lipids were dissolved in chloroform:methanol 2:1, and appropriate volumes were combined to obtain the corresponding mixtures in accordance with the different lipid compositions included in the orthogonal composite factorial design of Box-Behnken used (20). Lipid mixtures were then placed in culture tubes and the solvent was removed with a stream of nitrogen and finally under high vacuum at room temperature.

Aqueous dispersions of each Iipid mixtures were then prepared by suspension in buffer containing 100 mM NaCl, 5 mM TRIS (supplemented with 10 mM CF dye when studhing bilayer permeability and internal volume), to provide the final concentration of 1 mg lipid per ml and pH 7.50. The lipids were left to hydrate for 30 min under nitrogen with occasional shaking. Suspensions were then sonicated in a bathsonicator (Bransonic 12) at 80°C until the suspensions became clear, usually about 15 min.

To study the alterations in the bilayer permeability due to the presence of Triton X-100 nonionic surfactant and to determine the internai volume of these structures, vesicles containing CF were freed of unencapsulated fluorescent dye by passage through Sephadex G-50 medium resin (Pharmacia, Uppsala, Sweden) by column chromatography. The preparations were then annealed at 80°C for 30 min and incubated at 37°C under nitrogen atmosphere.

Characterization of Liposomes

Bilayer Lipid Composition

The different lipid compositions of vesicles were determined using Thin-layer chromatography (TLC) coupled to an automated ionization detection (FID) system (Iatroscan MK-5, Iatron Lab. Inc. Tokyo, Japan). This technique considerably improves the sensivity of TLC and ailows quantitation of separated compounds (22). This method has been used to quantify most kind of lipids from different sources (23).

Liposomes (lmg lipid/ml buffer) were directly spotted onto silica gel coated Chromarods (type S-III) in 0.5, 1, and 1.5μl aliquots using a semiautomatic sample spotter SES 3202/IS-02 with a precision two-microliter syringe. The rods (in sets of 10 mounted semi-permanently in stainless steal racks) were developed for a distance of 10 cm using solvent mixture i) n-hexane/ethyl ether/formic acid (5012010.3) to separe the non-polar lipids, **PA** and Chol from the rest of the compounds. A partial scan of 80% of the rods were performed to quantify and eliminate them. A redevelopment of Chromarods with chloroform/methanol/ammonia (5811012.5) twice for 7 cm lead to a good separation of the polar lipids, Cer and Chol-sulf from the buffer that remains on the spotting place. **A** total scan was performed to quantify Cer and Chol-sulf. The same procedure was applied to different standard solutions of **PA ,** Chol, Cer and Chol-sulf dissolved in chloroform:methanol 2:1 to obtain the calibration curves for the quantification of each compound.

Phase Transition Temperature by ^{1}H NMR studies

^{1}H NMR spectra were recorded on a Varian Unity of 300 MHz. The dispersed suspension was made from 1 mg of the mixture of lipids included in stratum comeum in 1 ml of D_2O (99.99% D) after 15 min of sonication in a bath sonicator (Bransonic 12) at 80°C. The NMR spectra were measured at different temperatures. The line widths of the CH, band at 1.3 ppm were measured. 1024 scans were accumulated each time.

Determination of particle size distribution and polydispersity of bilayers

Mean vesicle size distribution of liposome preparations, and their corresponding polydispersity indexes, defined as a measure of the width of the particle size distribution obtained from the "cumulants analysis", were determined at different lipid compositions. Previously, the vesicles containing CF were freed of unencapsulated material by a column chromatographic method. The determinations were carried out using a Photon correlator spectrometer (Malvern Autosizer 4700c PS/MV), by particle number measurement at 37°C and lecture angle of 90º.

Determination of permeability alterations of bilayers

The permeability alterations of different bilayers due to the presence of the nonionic surfactant Triton X-100 were determined by monitoring the release of the CF from these structures.

Liposomes containing concentrated CF in the interior of the vesicles hardly fluoresce, but fluorescence strongly increases on liberation from the concentration quenching when CF is released from the interior of the bulk aqueous phase. Therefore, permeability changes in liposomes caused by surfactants can be determined by monitoring the increase in fluorescence intensity of the liposome preparations due to the CF liberated (19,24). Fluorescence measurements were made with a Shimadzu RF-540 spectrofluorophotometer equipped with a thermoregulated cell compartment. On excitation at 495 nm, a fluorescence maximum emission of CF was obtained at 515.4 nm.

The general procedure to assess the effect of the surfactant on the release of liposomai contents consists on treating aliquots of liposome (4 ml) with a smail amount of buffered surfactant solution (150 μl of 1 mM Triton X-100). Afterwards a measure of the proportion of the fluorescent dye released was carried out. The amount of released CF was calculated by means of the following equation (19):

$$\%\text{CF release} = \frac{I_t - I_0}{I_\infty - I_0} \times 100 \qquad [1]$$

where I_0 is the initial fluorescence intensity of the CF-loaded liposome suspension at 515.4 nm in the presence of any surfactant and I_∞ is the fluorescence intensity at 515.4 nm after destroying the liposomes by addition of Triton X-100 (60μl of 10% (v/v) aqueous Triton X-100 solution to 4 ml of liposome suspensions). I, corresponds to the fluorescence intensity 60 minutes after adding Triton X-100 (150 μl of a 1 mM solution) to the liposome suspensions, being this period of time the minimum needed to obtain a constant rate of CF release in each case.

Internal Volume

The internal volume or "captured volume" is defined as the volume enclosed by a given amount of lipid and expressed as ml/mg lipid. This parameter depends on the radius of the liposome formed and hence is affected by the lipid composition of each vesicle, and the ionic composition of the medium. This parameter was determined from spectrofluorophotometric analysis of the CF trapped in liposome vesicles. To this end, liposome vesicles were freed

of unencapsulated dye by column chromatography and the CF concentration was determined after destroying the liposomes by addition of 10% (v/v) Triton X-100 aqueous solution.

Electron mieroscopy

An Hitachi H-600AB transmission electron microscope operated at 75 kv was used. Carbon-coated copper/palladium grids G-400 mesh, 0.5 Taab with 0.5 % Kollodium films E 950 in n-amylacetat were used. A drop of the vesicular solution was sucked off the grid and after 1 minute with filter paper down to a thin film. Negative staining with a drop of a 1 % solution of uranyl acetate was prepared. After 1 minute this drop was again removed with filter paper and the resulting staining film dried in a dust free place.

Statistical Treatments of Results

Experiences were planned according to a Box and Behnken experimental plan (20,25) for three variables. Variables were the following: X, = Ceramide (%), X_2 = Cholesterol(%) and X_3 = Palmitic acid (%). Having in mind that vesicles were formed by four components, a fourth additional variable to complete the 100% vesicles composition corresponding to Cholestheryl sulphate was considered X_4 = Cholestheryl sulfate (%), being

$X_4 = 100 - (X, + X_2 + X_3)$.

The Box and Behnken experimental plans are a kind of three level variable arrangements useful for estimating the coefficients in a second degree graduating polynomial. This design either meet, or approximately meet, the criterion of rotatability, and can be orthogonally blocked. Variable levels are shown in Table 1. Coded and uncoded levels of the variables for every experiment carried out are indicated in Table 2 and in Table 3 the results obtained are shown. From these results, the influence of the X, (Ceramide), X_2 (Cholesterol) and X_3 (Palmitic Acid) on the internal volume of vesicles , the particle size distribution, the polydispersity index as well as the percentage of CF released from liposomes were adjusted, by the following second order polynomial function:

$$Y = b_0 + \sum_i b_i X_i + \sum_{ij} b_{ij} X_i X_j \quad \text{for } i \geq j. \qquad [2]$$

where b_0 is the independent term, which depends on the mean value of the experimental plan, b_i are regression coefficients which explains the variables influence in its lineal form (increasing or decreasing tendences), and b_{ij} are regressiori coefficients which explains the variables influence in its quadratic form which could define optimum zones (maximums or minimums) and possibles interactions between them (synergism effects). Equation regression coefficients b_i, b_{ij}, determination coefficient R^2 (squared deviations from the mean explained by the equation), equation F-Snedecor coefficient with its signification level are shown in

Table 4. To remove from the equation the non significative terms the Stepwise selection procedure has been applied (20). Therefore beside every regression coefficient appears its signification level obtained from the t-Student test. The adjusted equations explain a great part of the response variation. In relation to the equation F-Snedecor values it can be said that ail of them have a great level of signification, that is to say that the probability to explain a non existent variation through the equation is lower than 0.1% for the internai volume, the particle size distribution, and the CF release, and lower than 1% for the polydispersity index.

Table 1

DESIGN VARIABLES	CODED LEVELS		
	-1	0	+1
X_1 = CERAMIDE	34%	40%	46%
X_2 = CHOLESTEROL	21.25%	25%	28.75%
X_3 = PALMITIC ACID	21.25%	25%	28.75%

Table 2

Exp. nr.	Coded levels			CERAMIDE (%)	CHOLESTEROL (%)	PALMITIC ACID (%)	CHOLEST. SULPHATE (%)
	X_1	X_2	X_3				
1	0	-1	-1	40	21.25	21.25	17.5
2	-1	0	-1	34	25	21.25	19.75
3	1	. 0	-1	46	25	21.25	7.75
4	0	. 1	-1	40	28.75	21.25	10
5	-1	-1	0	34	21.25	25	19.75
6	1	-1	0	46	21.25	25	7.75
7	0	0	0	40	25	25	10
8	0	0	0	40	25	25	10
9	0	0	0	40	25	25	10
10	-1	1	0	34	28.75	25	12.25
11	1	1	0	46	21.25	25	0.25
12	0	-1	1	40	21.25	28.75	10
13	-1	-1	1	34	21.25	28.75	16
14	1	0	1	46	25	28.75	0.25
15	0	1	1	40	28.75	28.75	2.5

Table 3

Exp. nr.	PERMEAB %	INTERNAL VOL %	P.DISP. INDEX %	PARTICLE SIZE (nm)
1	28.23	3.87	0.233	337.7
2	29.57	4.03	0.278	352.7
3	27.48	3.61	0.324	312.4
4	29.26	3.69	0.298	227.5
5	27.89	3.89	0.244	373.8
6	24.90	3.35	0.516	588.4
7	28.49	3.48	0.309	249.8
8	28.57	3.50	0.355	271.9
9	27.41	3.48	0.434	270.5
10	28.57	3.71	0.433	251.5
11	22.40	3.02	0.304	271.9
12	24.50	3.19	0.360	337.7
13	25.17	3.51	0.049	267.3
14	20.19	2.72	0.573	597.7
15	25.83	3.06	0.212	259.5

Table 4

Y Coef.	INTERNAL VOLUME	PARTICLE SIZE	POLYDISP. INDEX	PERMEA BILITY
b_0	3.49**	279.23**	0.3707**	27.928**
b_1	-0.298**	70.70*	0.0771*	-2.252**
b_2	-0.099**	-81.95**	-	-
b_3	-0.347**	-	-	-2.133**
b_{11}	-	92.67*	-	-1.530*
b_{22}	-	-	-	-0.807
b_{33}	-0.037·	-	-0.0678·	-
b_{12}	-0.045+	-41.45·	-0.0965·	-0.978+
b_{13}	-0.079*	102.80*	0.0954+	-1.169+
b_{23}	-	-	-	-
R^2	0.9945	0.9038	0.8064	0.9469
eq.F	241.0'-	16.91**	10.41-	23.97**
Remarks: ** 0.1%, * 1%, + 5%,. 10% significative levels.				

The level of controlled variation by the variables through the adjusted model is shown by the R^2 coefficient. Internai volume reaches the highest explanation level 99.45%. Particle size distribution and CF release reach the 90.38 and 94.69 % level of explanation and the lowest are the polydispersity indexes with an 80.64 %.

With these results it can be said that the lipid liposomes bilayer formation and their physico-chemicai properties through lipid composition can be accurately controlled.

RESULTS AND DISCUSSION

Liposome Formation *and* Characterization

Regarding the experimental planning of our study, in ail the 15 experiments done according the combination in relative percentages of the four components indicated in Table 2, the formation of liposome structures took place. This is a very important fact because each lipid component individuaily considered is unable to form liposomes. In fact, the most interesting feature of our work is to know from the physico-chemical point of view the synergistic cooperative behaviour of those molecules in order to promote bilayer formation.

Lipid Analysis *and* Phase Transition Temperature Determination

Every liposome composition investigated according to the optimizing model of Box-Behnken (lipid compositions given in Table 1), was directly spotted onto silica gel coated Chromarods in order to be anaiyzed by TLC/FID technique.

FID analysis of liposome vesicles, permited us first to quantify the PA and Chol leaving in the spotting place Cer and Chol-sulf and the buffer (Figure 1). A partial scan of 80% of the rods were performed to quantify and eliminate PA and Chol. After a redevelopment of the same rods, a good separation of Cer from Chol-sulf and from the buffer was achieved (Figure 2). Therefore, a total scan was performed to quantify Cer and Chol-sulf.

Previously, the same procedure was performed with lipid sîandard solutions obtaining the calibration curves to be applied to the quantification of each compound.

No concentration changes of any lipid compound were obtained after the liposome analysis being performed. These results imply that the liposomes studied on the optimizing model are in fact composed by the lipid amounts described in Table 1. With regards to the Phase Transition Temperature of different lipid mixtures, determinations were carried out using ^1H NMR. This parameter was, in ail cases, lower than 80°C (data not shown). Accordingly, ail the liposome suspensions were prepared by sonication at 80°C during 15 min.

FIGURE 1

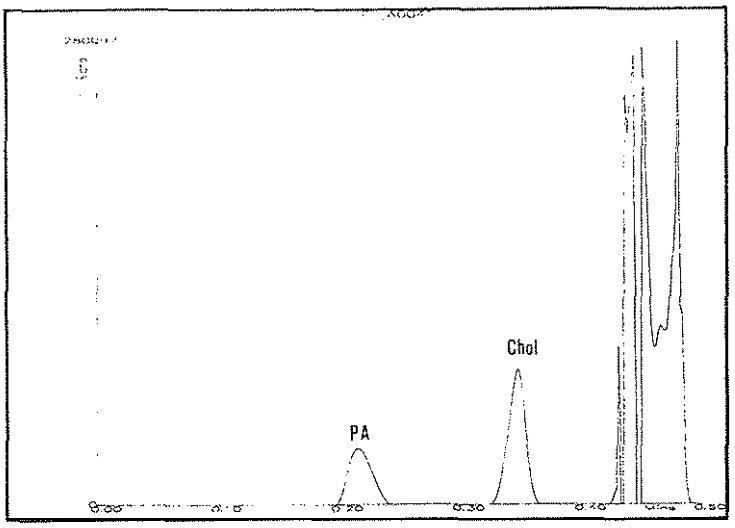
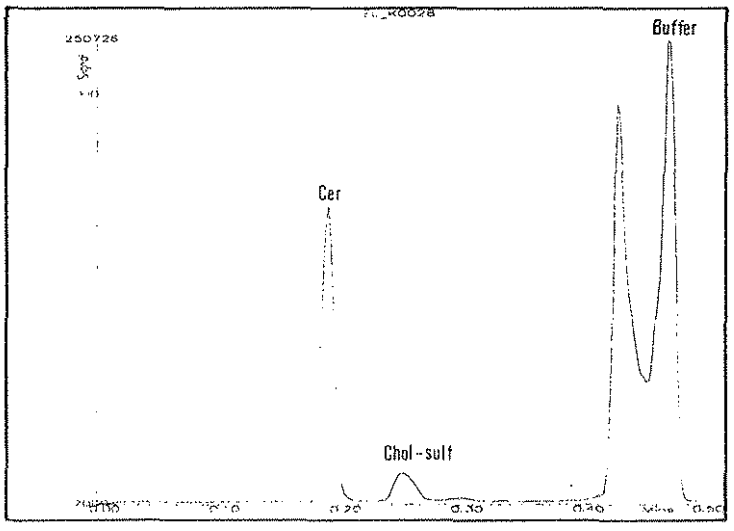


FIGURE 2



Internal Volume of Bilayers

Figure 3(A-C) shows the evolution of the internal volume (ml/mg iipid) of liposome suspensions, the response surface corresponding to the variation of Cer (X,) (from 34% to 46%) and PA (X_3) (from 21.25% to 28.75%) for three levels of the variable X_2, (Chol) 21.25% (Fig 3-A), 25% (Fig 3-B) and 28.75% (Fig 3-C).

As regards of the graphs plotted, it should be noted that increasing percentages of Cer and PA resulted in a decrease of the internal volume of vesicles. This response also diminished as the Chol percentage of bilayers increased. Thus, at the maximum relative concentrations of Cer and PA, the internal volume decreased aproximately from 3 ml/mg to 2.7 ml/mg when the Chol percentages increased from 21.25% (Fig 3-A) to 28.75% (Fig 3-C).

The surface response variations of the variable X_4 (Chol-sulf) with regards to the variations of Cer (X_1) and PA (X_3) for three levels of Chol (X,) are indicated in Figure 4(A-C).

Comparing Figures 3 and 4, it is noteworthy that theinfluence of the Chol-sulf in the

evolution of the internal volume of liposomes led to an opposite tendency than showed by the others components used. Thus, increasing concentrations of Chol-sulf resulted in a rise in the internal volume of bilayers, specially for the Chol level 28.75% (Figures 3-C), where the highest level (3.9 ml/mg lipid) was obtained for only 14% of Chol-sulf.

Vesicle size distribution *of vesicles* and polydispersity index

The evolution of the mean vesicle size distribution responses of liposomes varying the concentrations of Cer (X,) and PA (X,), the Chol concentration (variable X,) remaining constant at three levels are given in Figure 5. In general, increasing relative concentrations of Cer and PA resulted in an increment of the mean vesicle size distribution, although this response decreased from 650 *nm* to 400*nm nm* when Chol concentration increased from 21,25% to 28.75%. The minimum level was obtained in the area of to the central Cer and PA concentrations. Comparing Fig 4 and *5*, the increasing presence of Chol-sulf in bilayers also grew the size distribution of vesicles. Likewise, the presence of low Cer and high PA percentages led, in all cases, to the formation of low vesicle size suspensions.

In order to determine the statistical reliability of the vesicle size distribution responses, the evolution of the corresponding polydispersity indexes was also considered as a response in the statistical treatment. The curves obtained are plotted for the same variables and in the same range of relative concentrations in Figure 6. As Cer and PA relative concentrations rose the polydispersity indexes also increased reaching the highest vaiues for Chol concentration 21.25%. However, increasing Chol concentrations resulted in a diminution on these absolute values although increasing the area at which this parameter showed values higher than 0.35 (shaded area). Comparing the surface responses plotted in Figures 5 and 6, it may be observed that for 21.25 and 25% of Chol the areas corresponding to the highest vesicle size distribution also corresponded to the highest polydispersity indexes (shaded areas). Inversely, the lower the polydispersity indexes, the lower the vesicle size distribution of liposomes.

The global evaluation of the responses obtained for all the lipid compositions studied, leads to an apparent contradiction, specially when cornparing the evolution of the internal volume of vesicles with their corresponding size distribution. Thus, for increasing relative concentrations of Cer, PA or Chol, the smaller the internal volume, the greater the vesicle size distribution. This apparent contradiction should be explained by the formation in this conditions of aggregated structures, leading increased lipid structures with low internal volumes. This hypothesis should be supported by the increasing evolution of the polydispersity indexes observed in this conditions.

FIGURE 3 INTERNAL VOLUME (ml/mg lipid)

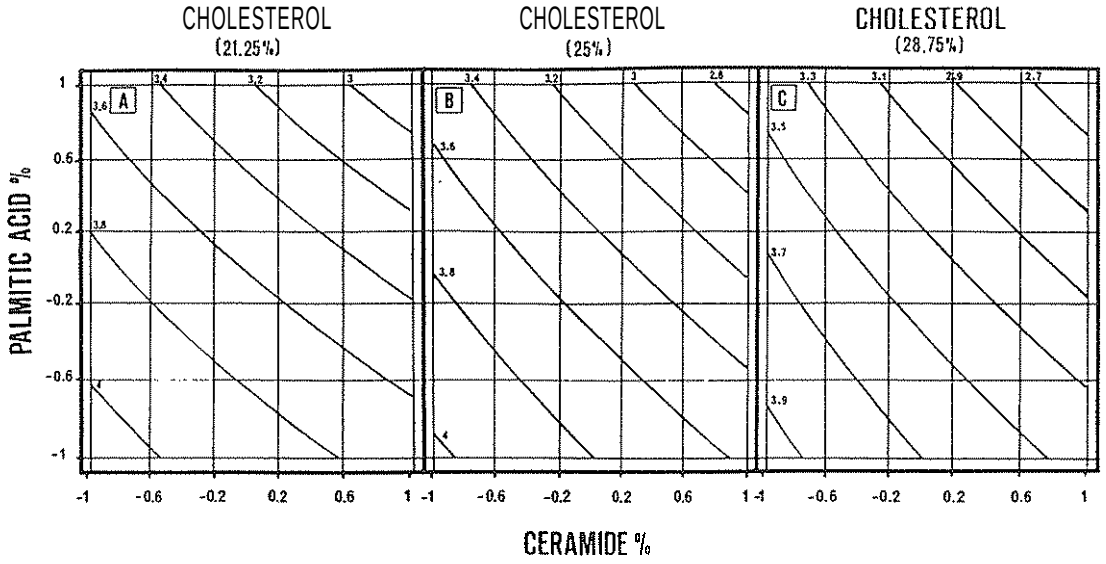


FIGURE 4 CHOLESTERYL SULFATE %

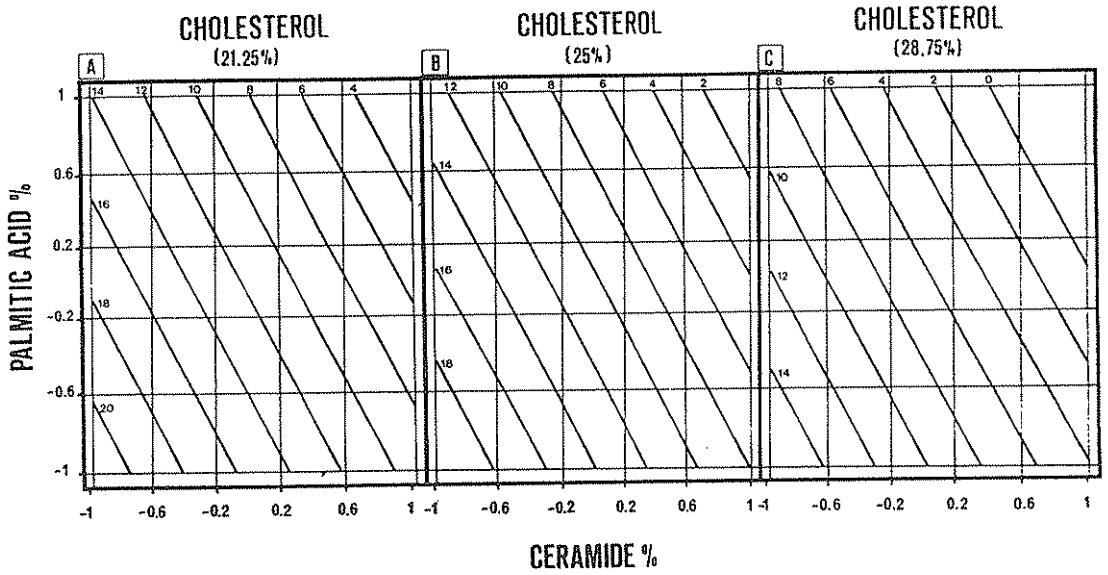
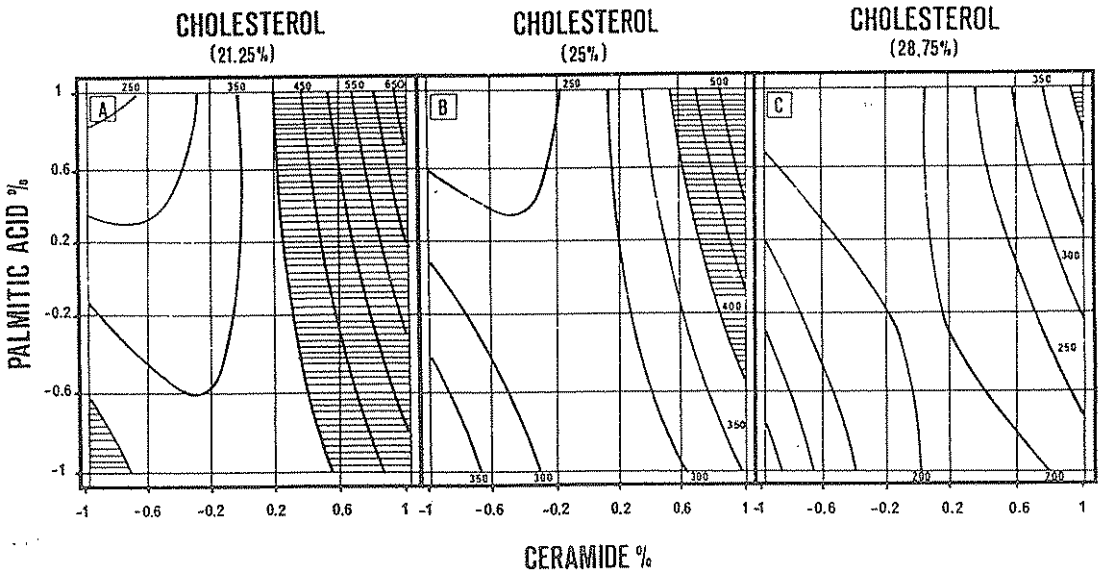


FIGURE 5 MEAN VESICLE SIZE (nm)



In order to verify this hypothesis, a series of microscopic observations of liposomes suspensions formed by different lipid mixtures were carried out using the transmission electron microscopy technique. To this end, liposome suspensions corresponding to the experiments 2, 7 and **14** (Table 2) were examined, and some representative microphotographs are shown in Figure 7. In ail cases, only unilamellar liposome structures were formed in agreement with the results early pubiished (15). Liposome suspensions corresponding to the experiment 14 showed vesicle suspensions with an irregular size distribution and showing signs of structural aggregation, whereas the expenment 7 presented more homogenwus vesicle distribution without aggregation. The microphotograph corresponding to the experiment 2 also showed irregularities in the vesicle size with a certain level of aggregation although this effect being lesser than those observed for the experiment 14. This observations confirm our hypothesis in relation to the aggregation of these structures both at high or low relative percentages (Exp 2 and **14)** of Chol-sulf, the central composition resulting more balanced with regards to the aggregation stability.

Permeability Studies

Bearing in mind that the stratum corneum lipids constitute the epidermal barner to water penetration in the mammalian epidermis, the influence of the lipid composition in the permeability alterations of these structures was studied. To this end, a kinetic study on the sub-solubilizing interaction between the stratum corneum lipid liposomes containing CF in the interior of vesicles and the nonionic surfactant Tnton X-100 was camed out to determine the time needed to obtain a constant rate of CF release of liposomes. The surfactant/liposome relative concentration was previously chosen to obtain, in all cases, bilayer permeability changes without alteration in the lamellar liposomal structure (26). From the results obtained, similar evolution in the permeability kinetics was obtained in all cases: about 60 min was needed to achieve a CF release equilibrium. As a consequence, the permeability studies were carned out 60 min after addition of surfactant to liposomes at 37°C. It is interesting to note that the percentages of CF release from lipid vesicles in the absence of surfactant 60 min after preparation of liposome suspensions were negligible.

Figure 8(A-C) shows the evolution of bilayer permeability of liposome suspensions after the addition of Triton X-100 buffered solution. The response surfaces showed also correspond to the same variables and in the same range of relative concentrations.

Regarding the surface responses it is noteworthy that the permeability of vesicles decreased as the relative bilayer proportion of Cer (X_1) and PA (X_3) increased reaching the minimum

FIGURE 6

POLYDISPERSITY

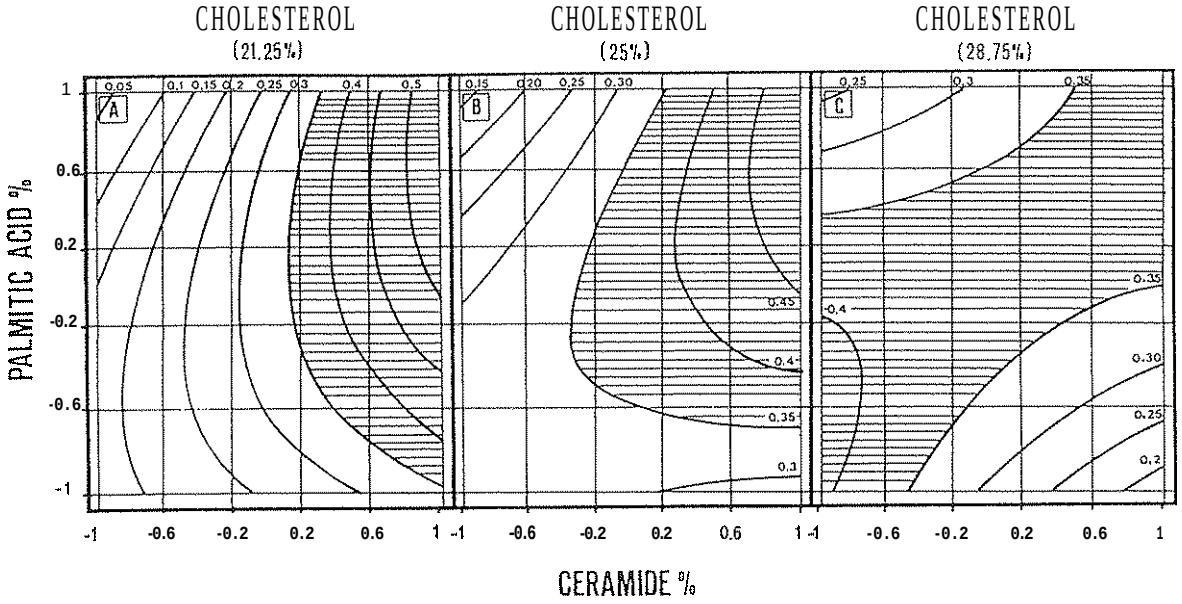


FIGURE 7

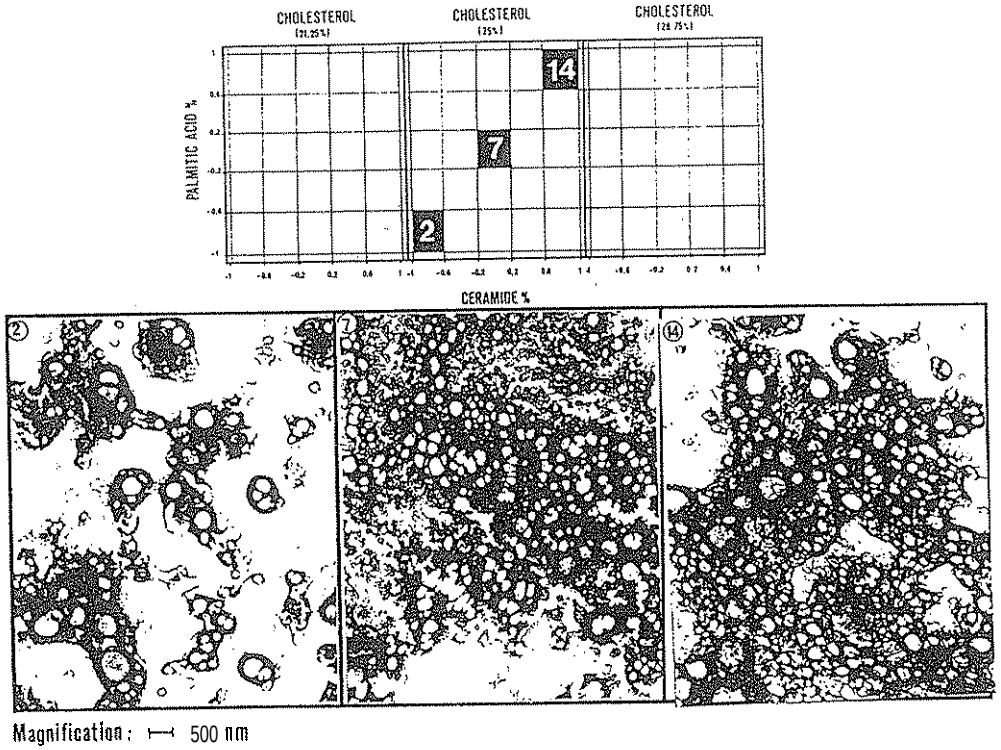
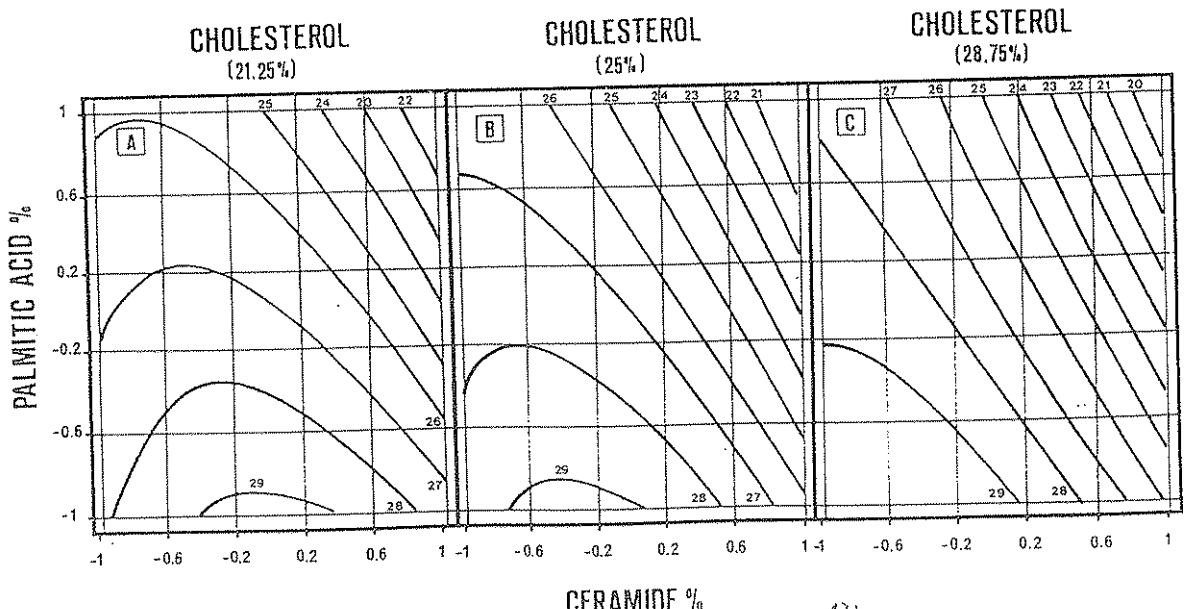


FIGURE 8

LIPOSOME PERMEABILITY (%)



7.- FINAL REMARKS AND FUTURE EXPECTATIVES

It is interesting to know more about the interaction of these liposomes with the skin surface and their interactions with or integration into the intercellular lamellae of the stratum corneum. We also need to know more about the incorporation into and release from these liposomes of drugs useful for the treatment of skin diseases.

With regard to technology, it is likely that naturally occurring phospholipids will be replaced by inexpensive synthetic, well-defined amphipathic lipids of varying chain length and phase transition temperature. Methods for the preparation of liposomes will continue to improve in terms of high drug-to-lipid mass ratios, narrow vesicle size distribution, stability under storage and freeze-dried formulations. Also, it can be expected a wider use of lipid-bound drugs inserted into the bilayers and less use of water-soluble drugs captured in the aqueous phase.

Indeed, an area where liposomes have already had signifiant impact is topical applications for skin care with cosmetics. Liposome-based cosmetics have, in fact, contributed to the field of liposomology by demonstrating that stable vesicles can be prepared on an industriai scale. Technology is thus well advanced and the non-invasive character of topical use coupled with sophisticated approaches to enhance absorption suggest that a variety of products for the treatment of eyes, skin and external mucosal tissues may be forthcoming. Nevertheless, in an area of growing concern with side effects, the potential of increasing the beneîït to risk ratio of topical drug formulations, even in cosmetics, looks most rewarding.

The cosmetic technology uses in a great extension a series of active ingredients whose efficacy must be checked scientifically in ail cases. Among the factors regarding liposomes that a deeper knowledge is necessary can be indicated the followings: the characteristics and suitability of entrapment into liposomes, improving the stability of vesicle structure, to know the parameters that rule out the release of an active compound in strategic and stereospecific target sites, the action mechanisms implicated with each active ingredient, the real possibilities existing for the incorporation of some appropriate enzymatic systems in liposomes in order to prevent and control the quali- and quantitative presence of free radicals, that are direct responsible of many phenomena engaged in skin aging, etc.

Probably, future trends will be focused on some aspects of Supramolecular Chemistry related with the implantation of some selective criteria on liposomes technology such as action stereospecificity, stability and efficacy of vesicle structures. With all of these approaches in mind, the use in cosmetics of molecular machines could be an splendid reality.

It is interesting to comment some aspects described by A. Meybeck on the evolution of liposome cosmetics (27). Capture is an anti-ageing gel for the face which is still a big commercial success after its launch. It has been followed by many other products among which Myosphere, introduced in 1987, was the first emulsion containing liposomes. And in 1990, the first liposome face cream for men was launched.

But liposomes are not restricted to facial care, and the first formulation for body care launched in 1987 was followed by many others claiming mostly slimming effects. Some sunscreen or self-tanning products were also introduced in 1988 and later. For haircare, however, it was not until 1989 tbat a liposome preparation appeared on the market, and only a few others have been launched since.

As far as make-up is concerned, the first product to claim the inclusion of liposomes was a powder in 1988. After that, a mascara and several foundations were introduced.

The most surprising products are probably some perfume formulae launched recently.

Concerning activity of liposomes, in several cases, encapsulation of polypeptides in liposomes has been shown to improve the stimulation of skin cells in culture. For instance, the growth activation of fibroblasts by elastin peptides was increased from 57% to 108% and that of collagen peptides from 41% to 87%.

Liposomes containing an extract of Morus alba had a 37% depigmenting activity, whereas the extract presented in gel form had no signifiant activity. Also, a 2% hydroquinone or a combination of 2% hydroquinone and 1% kojic acid, there was also a marked improvement of effect by encapsulation in liposomes.

Tyrosine does not accelerate tanning. However, when it is formulated in liposomes it has some activity in enhancing pigmentation. A similar beneficial effect of liposomes has been found also for isobutyl methyl xanthine.

Also, patents are described on liposome encapsulation of biochemical compounds of high molecular weight such as glycosaminglycanes (hyaluronic acid or condroitinsulphate), proteins (collagen, elastin or reticuline) and specific compounds (retinoic acid or epidermal growth factor). As it can be appreciated, the application of liposomes can be directed to the cosmetic field (external layers of epidermis), to dermatology (topical action centred, at the first time, in skin tissues) and to the wide field of clinical pharmacology.

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